

### **REMARKS/ARGUMENTS**

Reconsideration of this application is requested. This Amendment addresses the issues raised in the Office Action of January 20, 2010, a final rejection, and accompanies a Request for Continued Examination.

In order to expedite further examination claims 1 – 139 are now cancelled, this action being taken without disclaimer and without prejudice to re-introduce subject matter of any of the cancelled claims or pursue non-elected subject matter in continuation applications.

#### **Claim Clarity**

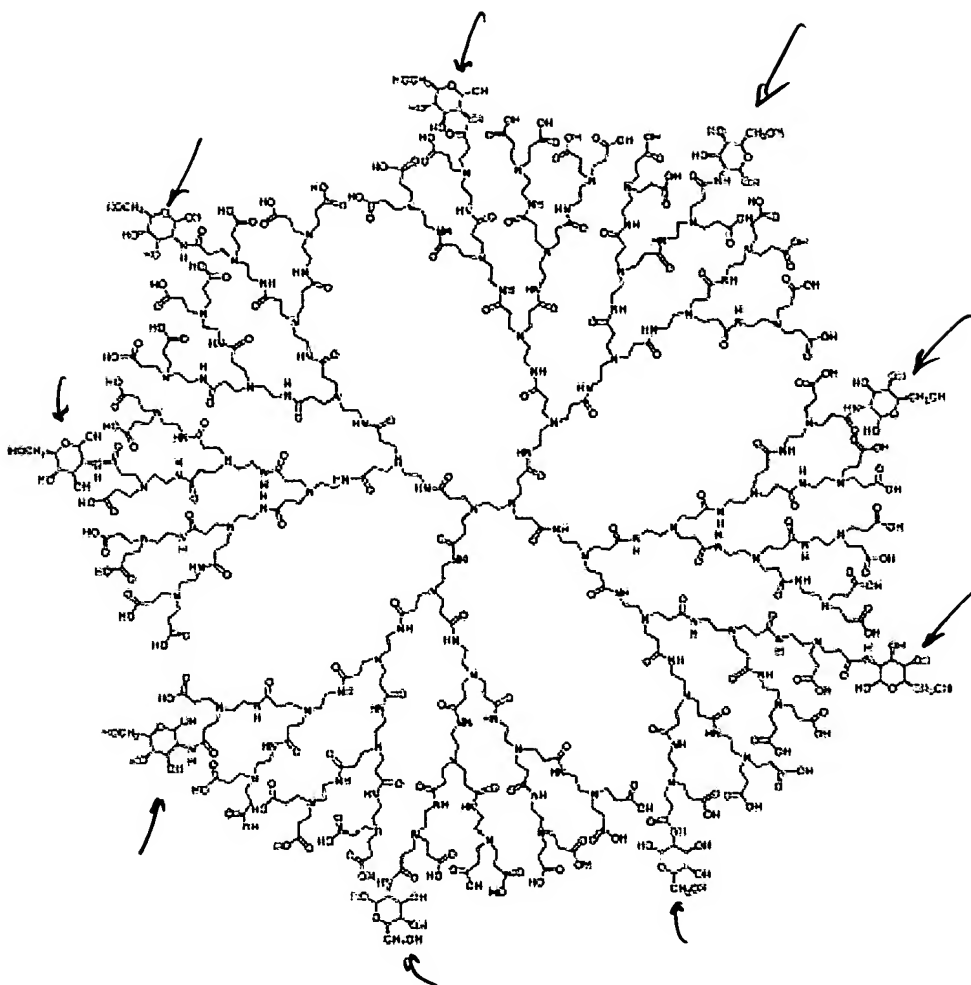
The claims have been amended and significantly narrowed to correspond very particularly to what has been contributed by the present inventors to the art. In particular, the claims as amended relate to only: anionic divergent synthesized cores of a particular size, with specific sugars and characterization of the desirable surface properties of the claimed molecules. Applicants submit that the inventors were clearly in possession of the invention at the time filing and the fact it was exemplified with only one or two dendrimer cores is not in itself in conflict with the requirements of 35 USC §112.

What is more, the function of the core is simply to support the sugars and the free carboxylic acids and at the time of filing the present application a plethora of divergent core dendrimers were available and described in the art for use in the present invention. Applicants submit that the Examiner's previous objection under 35 USC 112 is moot in light of the presently amended claim.

#### **35 USC §103**

#### **Technical background**

The application as filed contains Figure 1b which shows the PAMAN dendrimer core. This is not the final construct presently claimed. Rather, the glycodendrimer of the present invention can be represented as follows:



The sugar moieties conjugated to the dendrimer core are shown in red (arrows added in manuscript to accommodate monochromatic scanners). The claim has been amended to reflect that not all the terminal carboxylic positions on the dendrimer core are occupied by the sugar.

The presently claimed molecules have highly optimized properties and are believed to be special in the biological profile that they present. In particular **in addition to a therapeutic activity**:

they have very low toxicity,

they are suitable administration to human because the residual organic solvent content is low, and

they are not anti-coagulants.

All of the above properties are described in the application as filed.

The desirable therapeutic profile and other properties are thought to result from the size and more particularly the surface properties of the claimed glycodendrimers.

The Examiner is respectfully reminded that reference to “3.5 generation or less” in the claim means the dendrimer is prepared by a divergent synthesis. That is to say the dendrimers of the present invention grow exponentially outwards with the last step being the conjugation of the sugar moiety.

Molecules prepared convergently are different and distinguishable from those prepared divergently.

The Examiner correctly points out that Rockendorf teaches sugarcoated non-carbohydrate dendrimers. *See* page 204 “type A” figure. These dendrimers are characterized by a **complete outer shell that of sugars with no free terminal ends of the core supporting structure**. This type of dendrimer can only be prepared by a convergent synthesis. *See* Rockendorf page 218.

In a convergent synthesis the individual components of the dendrimer are prepared separately, for example on resin to allow control of the construction. The sugar moiety is introduced at early stage and the last step is to assemble the dendrimer.

As the Examiner points out, there can be huge differences in molecular weight and number surface groups of dendrimer. However, the claims are limited to dendrimers of certain generation/size and certain surface properties. When the term dendrimer is used **loosely to include convergently or divergently prepared dendrimers** then, yes, the art is unpredictable. However, the Applicants have very carefully limited to entities, which are characterized by the exemplified molecules.

The presently claimed dendrimers are NOT sugar-coated cores as disclosed in Rockendorf. Please see the figure shown above where there are **free amino acid groups on the core** which supports the sugars. Compare this now with the figure on page 204 of Rockendorf and in fact they are not truly similar.

The beneficial arrangement in the compounds of the present invention is not taught or suggested in Rockendorf or Malik alone or combination.

There is no suggestion or motivation provided from the cited art that compounds with surface characteristics of the presently claimed compounds would be beneficial or desirable. Applicants submit that under 35 USC §103 the presently claimed preparations are not obvious.

Attached by way of background is a Nature paper published by one of the inventors. The paper contains similar data to that contained in the application as filed but in addition contains *in*

*vivo* data in tissue scarring model. It is a nice summary of the work done on the specific glycodendrimers currently claimed. Surprisingly the presently claimed compounds able to inhibit scarring in an *in vivo* eye surgery model (*see* also application as published PCT page 7 line 32).


The Examiner is no doubt aware of the scientific scrutiny that articles for publication in Nature are subjected to before publication. The Examiner is requested to accept the information set out in this published document, without the need to prepare a declaration from the inventor. Nevertheless, if a declaration will expedite prosecution of this matter applicants are happy to oblige Examiner.

Reconsideration and favorable action are solicited. Should the examiner require further information please contact the undersigned.

Respectfully submitted,

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# Polyvalent dendrimer glucosamine conjugates prevent scar tissue formation

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Dendrimers are hyperbranched macromolecules that can be chemically synthesized to have precise structural characteristics. We used anionic, polyamidoamine, generation 3.5 dendrimers to make novel water-soluble conjugates of D(+)-glucosamine and D(+)-glucosamine 6-sulfate with immuno-modulatory and antiangiogenic properties respectively. Dendrimer glucosamine inhibited Toll-like receptor 4-mediated lipopolysaccharide induced synthesis of pro-inflammatory chemokines (MIP-1 $\alpha$ , MIP-1 $\beta$ , IL-8) and cytokines (TNF- $\alpha$ , IL-1 $\beta$ , IL-6) from human dendritic cells and macrophages but allowed upregulation of the costimulatory molecules CD25, CD80, CD83 and CD86. Dendrimer glucosamine 6-sulfate blocked fibroblast growth factor-2 mediated endothelial cell proliferation and neoangiogenesis in human Matrigel and placental angiogenesis assays. When dendrimer glucosamine and dendrimer glucosamine 6-sulfate were used together in a validated and clinically relevant rabbit model of scar tissue formation after glaucoma filtration surgery, they increased the long-term success of the surgery from 30% to 80% ( $P = 0.029$ ). We conclude that synthetically engineered macromolecules such as the dendrimers described here can be tailored to have defined immuno-modulatory and antiangiogenic properties, and they can be used synergistically to prevent scar tissue formation.

Dendrimers are hyperbranched synthetic macromolecules that can be made using controlled sequential processes to give them defined structural and molecular weight characteristics. This endows them with a structural precision that is similar to low molecular weight molecules<sup>1,2</sup>. Dendrimers have been evaluated for biomedical applications that include drug delivery and the conjugation of pharmacologically active compounds<sup>3</sup>.

There is increasing recognition of the importance of polyvalent receptor-ligand interactions between carbohydrates and proteins in many aspects of cell surface-mediated immuno-regulation<sup>4,5</sup>. Therefore, any new medicine that aims to effectively modulate these interactions must possess multiple and cooperative receptor binding properties. Previous attempts to pharmacologically manipulate these interactions with synthetic linear polymers were impaired by the structural heterogeneity of the polymers and by their ability to activate complement and coagulation pathways<sup>6,7</sup>.

The chemical functionality of dendrimer end groups can be modified to make molecules with novel biological properties that exploit polyvalent and cooperative receptor-ligand interactions<sup>8,9</sup>. Whereas dendrimers with cationic end groups are often toxic after repeated intravenous use or topical ocular application<sup>10,11</sup>, anionic dendrimers are not<sup>12</sup>. This led us to postulate that anionic dendrimer conjugates of aminosaccharides and sulfated aminosaccharides

could be made via manipulation of their end groups to have defined immuno-modulatory and antiangiogenic properties, respectively. In this study, we describe two optimized conjugates that were safely used in combination to synergistically prevent scar tissue formation after surgery.

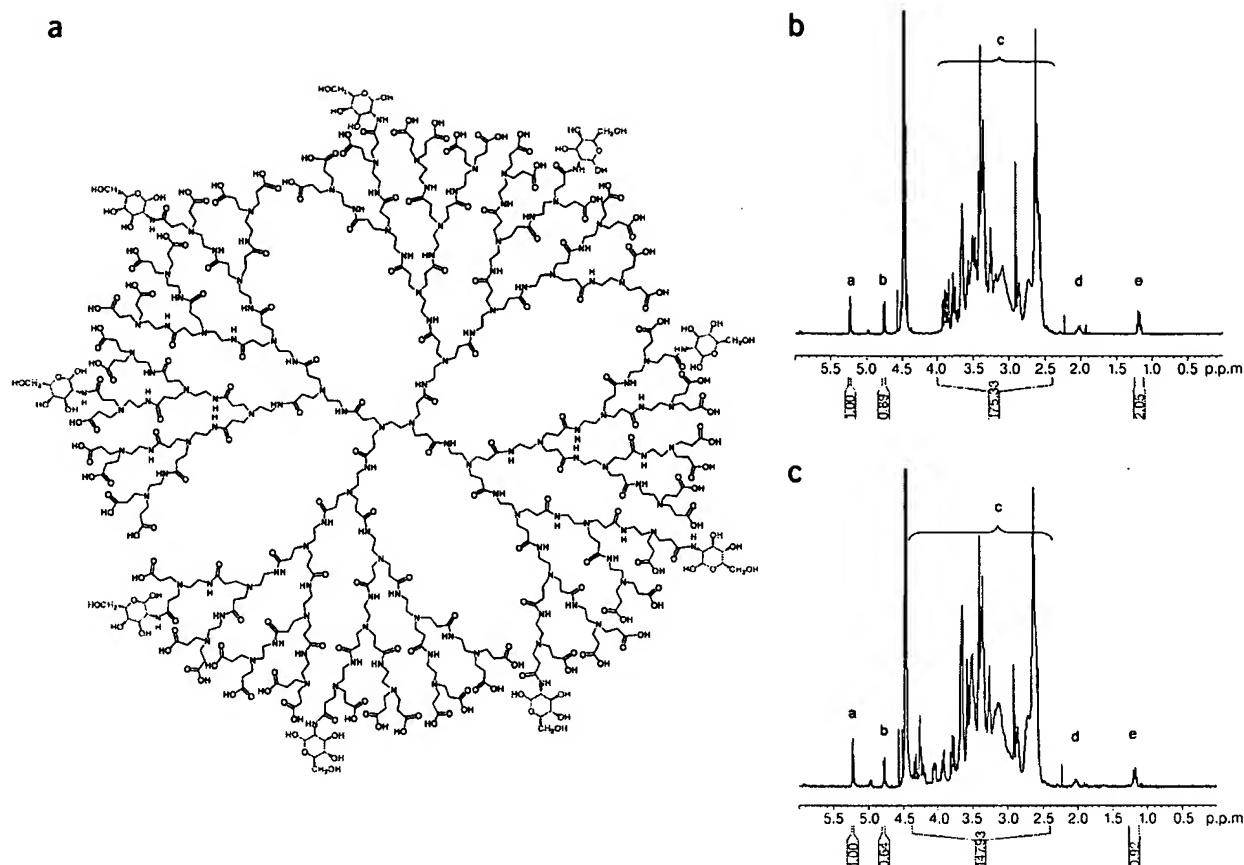
## RESULTS

### Synthesis of glycoconjugates

We conjugated the aminosaccharides glucosamine and glucosamine 6-sulfate to a generation 3.5 polyamidoamine (PAMAM) dendrimer (Dendritech) that had 64 carboxylic acid end groups. These two dendrimer glycoconjugates, dendrimer glucosamine (Fig. 1a) and dendrimer glucosamine 6-sulfate, were prepared with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDCI) at pH 5 to minimize *N*-acyl isourea formation. The total aminosaccharide conjugation was estimated by <sup>1</sup>H-NMR in D<sub>2</sub>O (Fig. 1b,c). The integral for both anomeric forms of the saccharide C-1 proton (C-1 H <sub>$\alpha$</sub>  and H <sub>$\beta$</sub>  signals at 5.2 p.p.m. and 4.8 p.p.m. respectively for both dendrimer glucosamine and dendrimer glucosamine 6-sulfate) was used as the diagnostic signal for the covalently bound saccharide. From the integrals of the remaining signals (2.4 to 4.0 p.p.m. for dendrimer glucosamine and 2.4 to 4.4 p.p.m. for dendrimer glucosamine 6-sulfate), we deduced the integral for one proton of

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**Figure 1** Polyvalent dendrimer glucosamine conjugates. (a) Representative structure of protonated, generation 3.5 PAMAM dendrimer glucosamine. (b,c) <sup>1</sup>H NMR spectra (D<sub>2</sub>O) for dendrimer glucosamine (b) and dendrimer glucosamine 6-sulfate (c). a, C-1 H<sub>α</sub> aminosaccharide; b, C-1 H<sub>β</sub> aminosaccharide; c, 6H aminosaccharide + 12H urea + 740H dendrimer; d, 2H urea, -CH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>-; e, 3H urea, CH<sub>3</sub>CH<sub>2</sub>-.

dendrimer (Supplementary Notes online). We used the ratio of aminosaccharide to dendrimer to determine the conjugation loading and expressed it as a percentage of the 64 carboxylic acid groups. The percentage loading of glucosamine and glucosamine 6-sulfate on the dendrimer was 14%. A total of 20 batches each of dendrimer glucosamine (MW = 13.6 kDa) and dendrimer glucosamine 6-sulfate (MW = 14.0 kDa) were made. Each batch was characterized before pooling them for detailed biological evaluation. The conjugates were stable after 12 month storage at 4 °C.

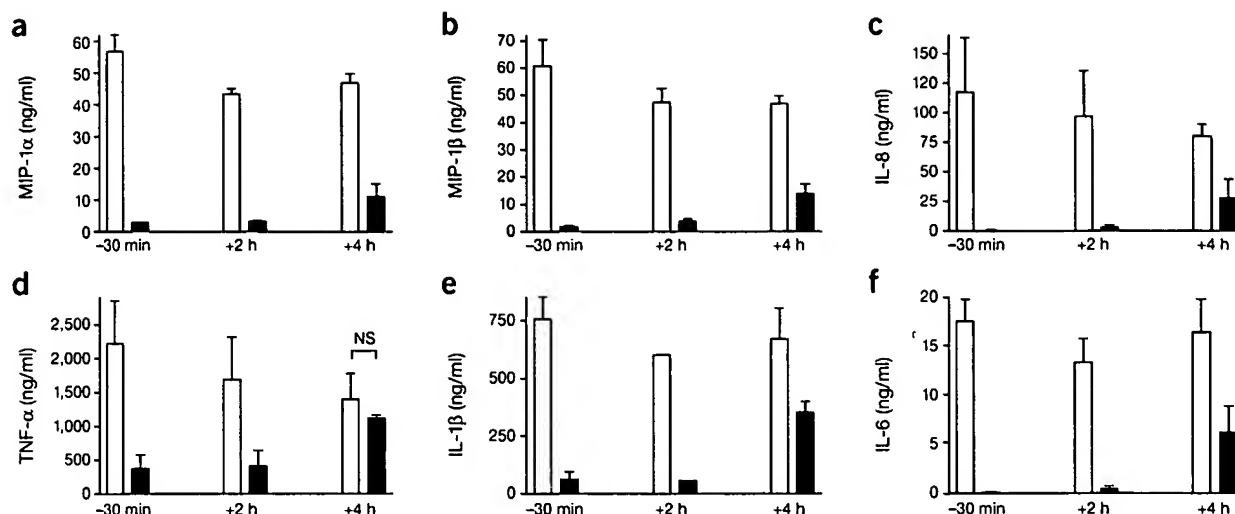
#### Cytotoxicity of glycoconjugates

The 50% lethal dose (LD<sub>50</sub>) of dendrimer glucosamine for the T cell line, SupT1, was 1,825 µg/ml (134 µM) ± 227 µg/ml and for the macrophage cell line U937, 2,837 µg/ml (209 µM) ± 107 µg/ml. The LD<sub>50</sub> of dendrimer glucosamine 6-sulfate for SupT1 cells was 307 µg/ml (22 µM) ± 29 µg/ml and for U937 cells, 271 µg/ml (19 µM) ± 19 µg/ml. When peripheral blood mononuclear cells (PBMCs), monocyte-derived macrophages (MDMs), immature monocyte-derived dendritic cells (DCs), CD3+ T lymphocytes and human umbilical vein endothelial cells (HUVECs) were cultured with dendrimer glucosamine (200 µg/ml) or dendrimer glucosamine 6-sulfate (100 µg/ml) for 5 d, we saw no adverse effect on cell viability or growth. Unlike other sulfated macromolecules, dendrimer glucosamine 6-sulfate had no anticoagulant, heparin-like or complement-activating activity when it was added to fresh whole blood<sup>6,13</sup>.

#### Inhibition of LPS cytokine release by dendrimer glucosamine

Preliminary experiments confirmed that dendrimer glucosamine and dendrimer glucosamine 6-sulfate did not alter the spontaneous release of chemokines (that is, MIP-1α and MIP-1β) or cytokines (that is, TNF-α and IL-1β) from resting PBMCs, MDMs or DCs. We then stimulated these cells with lipopolysaccharide (LPS; range 0.05–100 ng/ml) and found that 10 ng/ml caused the maximum release of MIP-1β and TNF-α into culture supernatants over a period of 21 h without inducing cellular toxicity<sup>14</sup>. *Salmonella minnesota* LPS was used because it acts through Toll-like receptor (TLR) 4 on macrophages and DCs, and because cDNA microarray studies of human monocytes and DCs have defined its sequential pattern of pro-inflammatory chemokine followed by cytokine release<sup>15,16</sup>.

Single donor PBMCs were then cultured with dendrimer glucosamine for 30 min followed by LPS for 21 h. There was a large and significant reduction in the release of the pro-inflammatory chemokines macrophage inflammatory protein (MIP)-1α (CCL3), MIP-1β (CCL4) and interleukin (IL)-8 (CXCL8) (Fig. 2a–c), and the pro-inflammatory cytokines tumor necrosis factor (TNF)-α, IL-1β and IL-6 (Fig. 2d–f). When we repeated this experiment by first exposing PBMCs to LPS and then adding dendrimer glucosamine 2 h or 4 h later, a significant inhibition of pro-inflammatory mediator release was still seen; the only exception was TNF-α when dendrimer glucosamine was added 4 h after the LPS (Fig. 2d). The 50%



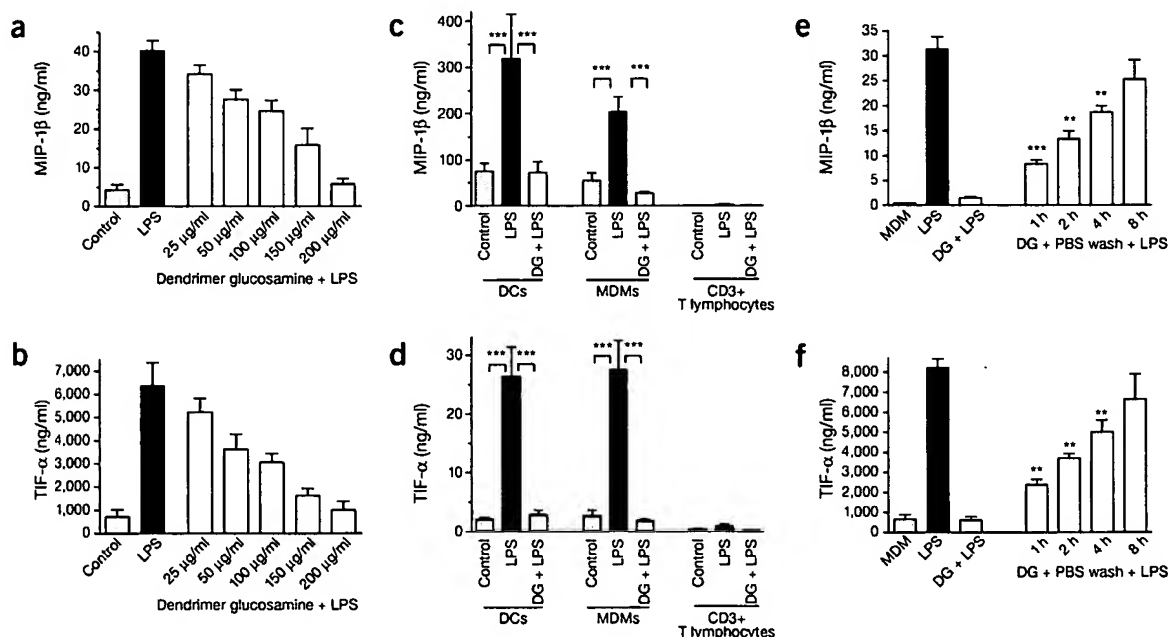
**Figure 2** Inhibitory effect of dendrimer glucosamine on LPS-mediated chemokine and cytokine release (a–f) Dendrimer glucosamine-inhibited pro-inflammatory chemokine (MIP-1 $\alpha$ , MIP-1 $\beta$ , IL-8) (a–c) and cytokine (TNF- $\alpha$ , IL-1 $\beta$ , IL-6) (d–f) release from human PBMCs when added 30 min before, or 2 h or 4 h after LPS.  $n = 3$ ;  $P < 0.001$ . White bars, PBMCs + LPS; black bars, PBMCs + dendrimer glucosamine + LPS. PBMCs, peripheral blood mononuclear cells; LPS, lipopolysaccharide; DG, dendrimer glucosamine; NS, not significant  $P > 0.05$ .

inhibitory concentration ( $IC_{50}$ ) of dendrimer glucosamine was  $92 (6.8 \mu M) \pm 15 \mu g/ml$  ( $n = 4$ ) (Fig. 3a,b). In further experiments, we used purified populations of cells to demonstrate that the primary effect of dendrimer glucosamine was on DCs and MDMs (Fig. 3c,d and Table 1), and that its immuno-modulatory activity was reversible (Fig. 3e,f). Neither the dendrimer intermediate formed in

the presence of the coupling agent only, nor glucosamine, nor the dendrimer had biological activity.

#### Inhibition of DC-mediated functions by dendrimer glucosamine

We then examined the ability of dendrimer glucosamine to interfere with DC maturation, activation and function. Treatment with



**Figure 3** Cell type specific effect of dendrimer glucosamine. (a,b) Dose-dependent inhibition of MIP-1 $\beta$  and TNF- $\alpha$  release from PBMCs when dendrimer glucosamine was added to human PBMCs for 1 h before LPS. The  $IC_{50}$  was  $92 (6.8 \mu M) \pm 15 \mu g/ml$  ( $n = 4$ ). Gray bars, PBMCs; black bars, PBMCs + LPS; white bars, PBMCs + dendrimer glucosamine + LPS. (c,d) Dendrimer glucosamine inhibited LPS-mediated release of MIP-1 $\beta$  and TNF- $\alpha$  from DCs and MDMs.  $n = 4$ ; \*\*\*  $P < 0.001$ . Gray bars, cells; black bars, cells + LPS; white bars, cells + dendrimer glucosamine + LPS. (e,f) The effect of dendrimer glucosamine on LPS-mediated release of MIP-1 $\beta$  and TNF- $\alpha$  from MDMs was reversible. MDMs were cultured with dendrimer glucosamine for 1 h, washed with PBS and LPS was then added 1, 2, 4 or 8 h later for a period of 21 h.  $n = 4$ ; \*\*  $P < 0.01$ ; \*\*\*  $P < 0.001$ . Gray bars, cells; black bars, cells + LPS; white bars, cells + dendrimer glucosamine + PBS wash + LPS. PBMCs, peripheral blood mononuclear cells; LPS, lipopolysaccharide; DG, dendrimer glucosamine; DC, dendritic cells; MDM, monocyte-derived macrophages.

**Table 1** Effect of dendrimer glucosamine on LPS-induced pro-inflammatory chemokine and cytokine mRNA synthesis in MDMs

| Chemokines and cytokines | MDMs <sup>a</sup>  | MDMs + LPS <sup>a</sup> | MDMs + DG + LPS <sup>a</sup> |
|--------------------------|--------------------|-------------------------|------------------------------|
| MIP-1 $\alpha$           | 14,438 $\pm$ 1,438 | 178,375 $\pm$ 6,125     | 21,925 $\pm$ 1,450           |
| MIP-1 $\beta$            | 13,938 $\pm$ 1,563 | 328,750 $\pm$ 11,750    | 30,125 $\pm$ 625             |
| IL-8                     | 50,125 $\pm$ 1,625 | 222,250 $\pm$ 750       | 101,250 $\pm$ 5,750          |
| TNF- $\alpha$            | 8,838 $\pm$ 413    | 122,375 $\pm$ 5,875     | 18,813 $\pm$ 1,313           |
| IL-1 $\beta$             | 1,163 $\pm$ 213    | 183,125 $\pm$ 33,875    | 10,588 $\pm$ 263             |
| IL-6                     | 1,575 $\pm$ 100    | 61,125 $\pm$ 13,375     | 3,650 $\pm$ 150              |

Results determined by quantitative, real-time Lightcycler PCR at 2 h.

<sup>a</sup>Copies/ $\mu$ g RNA ( $n = 4$ ). MDM, monocyte-derived macrophages; LPS, lipopolysaccharide; DG, dendrimer glucosamine.

dendrimer glucosamine did not interfere with LPS-induced maturation of DCs as determined by FACS analysis for CD25, CD80, CD83 and CD86 (Fig. 4a), or with  $\beta$ -interferon production (DCs + LPS 745  $\pm$  65 pg/ml versus DCs + dendrimer glucosamine + LPS 940  $\pm$  35 pg/ml;  $n = 2$ ,  $P = 0.16$ ; DCs alone 445  $\pm$  25 pg/ml). However, it did inhibit lymphocyte proliferation in the mixed leucocyte reaction (MLR) (Fig. 4b) and prevent  $\gamma$ -interferon production (206  $\pm$  24 versus 3  $\pm$  1 pg/ml;  $n = 3$ ,  $P < 0.001$ ). This effect was dose dependent with an IC<sub>50</sub> of 69  $\mu$ g/ml (5.1  $\mu$ M)  $\pm$  11  $\mu$ g/ml (Fig. 4c).

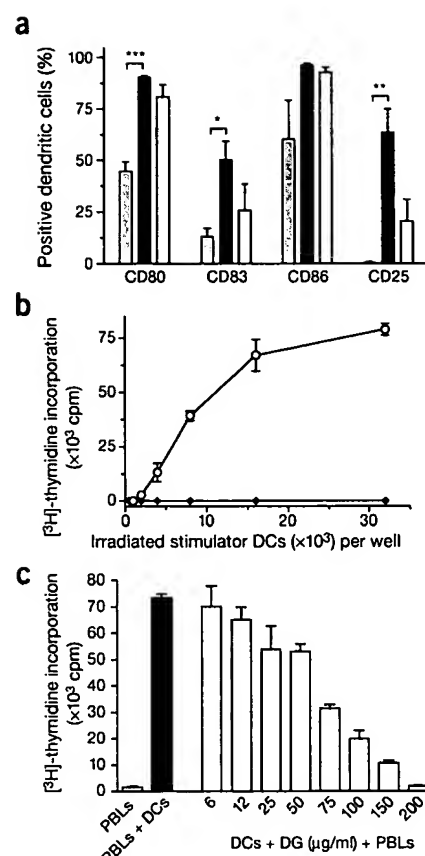
### The antiangiogenic activity of dendrimer glucosamine 6-sulfate

Dendrimer glucosamine 6-sulfate inhibited fibroblast growth factor-2 (FGF-2)-mediated proliferation of HUVECs by 50%  $\pm$  3% at 50  $\mu$ g/ml (3.6  $\mu$ M) but had no effect on vascular endothelial growth factor (VEGF)-mediated proliferation of HUVECs (Fig. 5a). Its effect on new human blood vessel formation was also determined. In the Matrigel assay, dendrimer glucosamine 6-sulfate completely inhibited new microtubule formation by HUVECs at 100  $\mu$ g/ml (7.1  $\mu$ M) (Supplementary Table 1 online). In the human placental angiogenesis assay, it reduced new blood vessel formation by 50% at 50  $\mu$ g/ml (3.6  $\mu$ M) with the first significant effect seen at day 18. Maximum inhibition was seen at day 28 (Fig. 5b). Neither the dendrimer intermediate formed in the presence of the coupling agent only, nor glucosamine 6-sulfate nor the dendrimer had biological activity.

### Therapeutic evaluation of dendrimer glucosamine conjugates

We used a validated and clinically relevant animal model of wound healing to establish whether an immuno-modulator (dendrimer glucosamine) and an antiangiogenic molecule (dendrimer glucosamine 6-sulfate) could act safely and synergistically to prevent scar tissue formation when used together (Fig. 5c). Subconjunctival scarring after glaucoma filtration surgery was chosen because (i) the surgical intervention is precisely defined (ii) it is one of the most aggressive models of excessive scar tissue formation available and (iii) surgical failure is due to excessive scar tissue formation. This animal model is better validated for scar formation in man than any other model of scarring<sup>17,18</sup>; drug efficacy in this model has previously led to two new treatments that were effective in patients undergoing glaucoma surgery<sup>19</sup>, and to treatments for other scarring situations in man<sup>20</sup>.

Glaucoma is the major cause of irreversible blindness worldwide. This surgical treatment is used in patients with glaucoma to create a new channel to drain aqueous humor from the eye. The subsequent reduction in intraocular pressure prevents the optic nerve damage that leads to blindness<sup>21</sup>. Post-surgical scarring is due to a persistent inflammatory and angiogenic response that promotes fibroblast

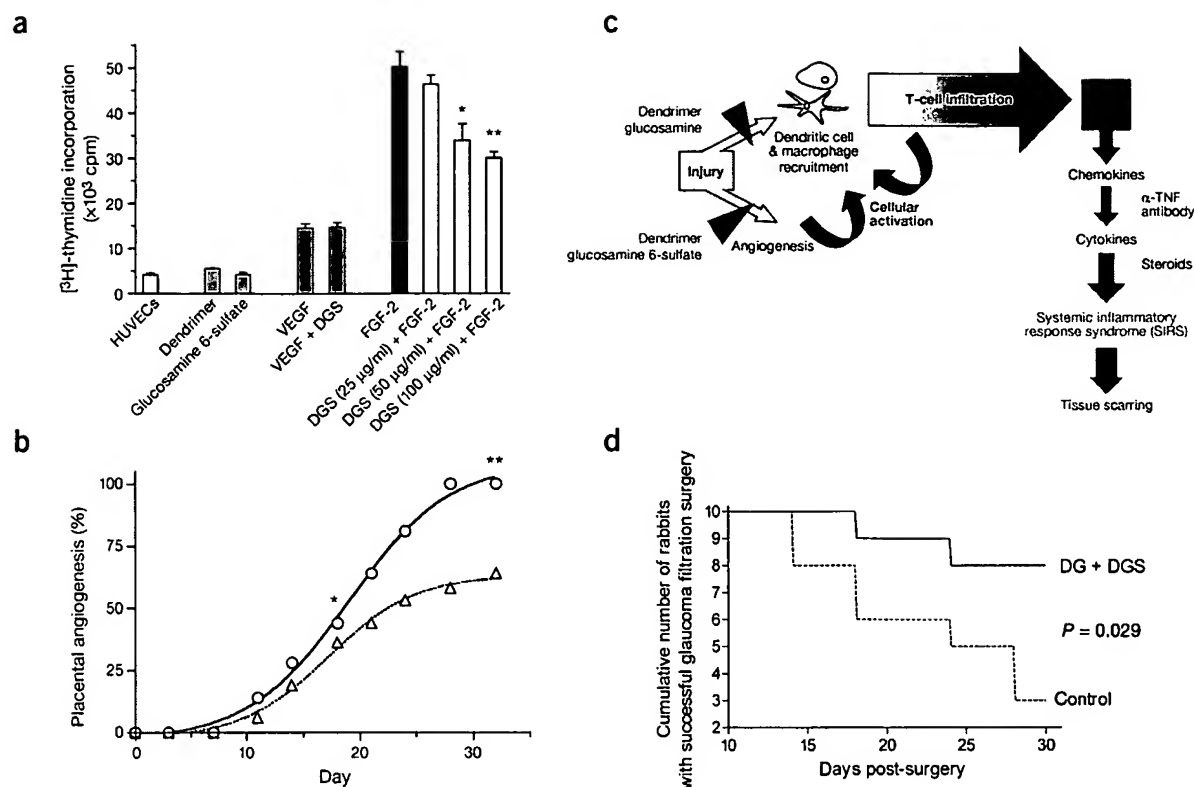


**Figure 4** Effect of dendrimer glucosamine on dendritic cell-mediated functions. (a) LPS-induced expression of the cell surface-associated molecules CD80, CD83, CD86 & CD25 was not affected by dendrimer glucosamine.  $n = 3$ ; \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ . Gray bars, DCs; black bars, DCs + LPS; white bars, DCs + dendrimer glucosamine + LPS. (b) The MLR was inhibited by dendrimer glucosamine.  $n = 3$ ,  $P < 0.001$ . (○) DCs + PBLs; (◆) DCs + dendrimer glucosamine + PBLs. (c) The MLR dose-response curve for dendrimer glucosamine. The ratio of DCs/PBLs was 1:4. There was a reversible and dose-dependent effect of dendrimer glucosamine on the MLR with an IC<sub>50</sub> of 69 [5.1  $\mu$ M]  $\pm$  11  $\mu$ g/ml. ( $n = 4$ ). Gray bars, PBLs; black bars, PBLs + DCs; white bars, PBLs + dendrimer glucosamine + DCs. PBLs, peripheral blood responder lymphocytes; LPS, lipopolysaccharide; DG, dendrimer glucosamine; DC, dendritic cells; MLR, mixed leucocyte reaction.

proliferation<sup>22</sup>. This problem has led to the widespread use of the anticancer drugs 5-fluorouracil and mitomycin C to try and minimize scar tissue formation. However, their clinical use leads to sight-threatening complications that include thin, leaking tissues and severe infections<sup>23</sup>.

We used the validated end point of bleb survival as our primary efficacy end point because this means that scar tissue formation has been significantly reduced and because this is indicative of the long-term patency of the new filtration pathway created (Supplementary Fig. 1 online). Combination treatment with dendrimer glucosamine and dendrimer glucosamine 6-sulfate increased the long-term success of the surgery from 30% to 80% ( $P = 0.029$ , Fig. 5d). The total dose of dendrimer glucosamine and dendrimer glucosamine 6-sulfate administered to each animal was 60.30 mg and 30.15 mg respectively. We saw no clinical, hematological or biochemical (including blood glucose) toxicity, and there were no local or systemic bacterial, viral or fungal infections in 30 rabbits treated over





**Figure 5** The anti-angiogenic activity of dendrimer glucosamine 6-sulfate. (a) Effect of dendrimer glucosamine 6-sulfate on VEGF and FGF-2-mediated proliferation of HUVECs. Dendrimer glucosamine 6-sulfate inhibited FGF-2-mediated proliferation of HUVECs by  $50 \pm 3\%$  at  $50 \mu\text{g/ml}$  ( $3.6 \mu\text{M}$ ).  $n = 5$ ;  $*P < 0.05$ ,  $**P < 0.01$ . (b) Dendrimer glucosamine 6-sulfate reduced the rate of new vessel formation in a human placental angiogenesis assay. Dendrimer glucosamine 6-sulfate (O). Media control ( $\Delta$ ). The results shown are for dendrimer glucosamine 6-sulfate at  $50 \mu\text{g/ml}$  ( $3.6 \mu\text{M}$ ). The graph is representative of two experiments done in quadruplicate. The degree of new vessel formation was quantified blind twice a week using a visual analog scale in which 0 = no growth, 1 = minimal new vessel formation, 2 = significant new vessel formation, and 3 = dense new vessel formation<sup>6</sup>. An angiogenesis score was derived from each count by dividing the total score (that is, the sum of all the wells) by the maximum possible score and then expressing the result as a percentage. A significant reduction in new vessel formation was first seen at day 18 ( $*P < 0.05$ ). Maximum inhibition was seen at day 28 ( $**P < 0.01$ ). (c) Illustration of the pathogenic mechanisms responsible for the systemic inflammatory response syndrome (SIRS) and scar tissue formation. The downstream sites of action of steroids and anti-TNF- $\alpha$  antibody are contrasted with the upstream sites of action of dendrimer glucosamine and dendrimer glucosamine 6-sulfate. (d) Cumulative graph of the number of rabbits with successful glaucoma filtration surgery.  $P = 0.029$  (log rank test). Bleb survival at day 30 is indicative of the long-term patency of the surgical filtration pathway created<sup>50</sup>. HUVECs, human umbilical vein endothelial cells; DG, dendrimer glucosamine; DGS, dendrimer glucosamine 6-sulfate; VEGF, vascular endothelial growth factor; FGF-2, fibroblast growth factor-2.

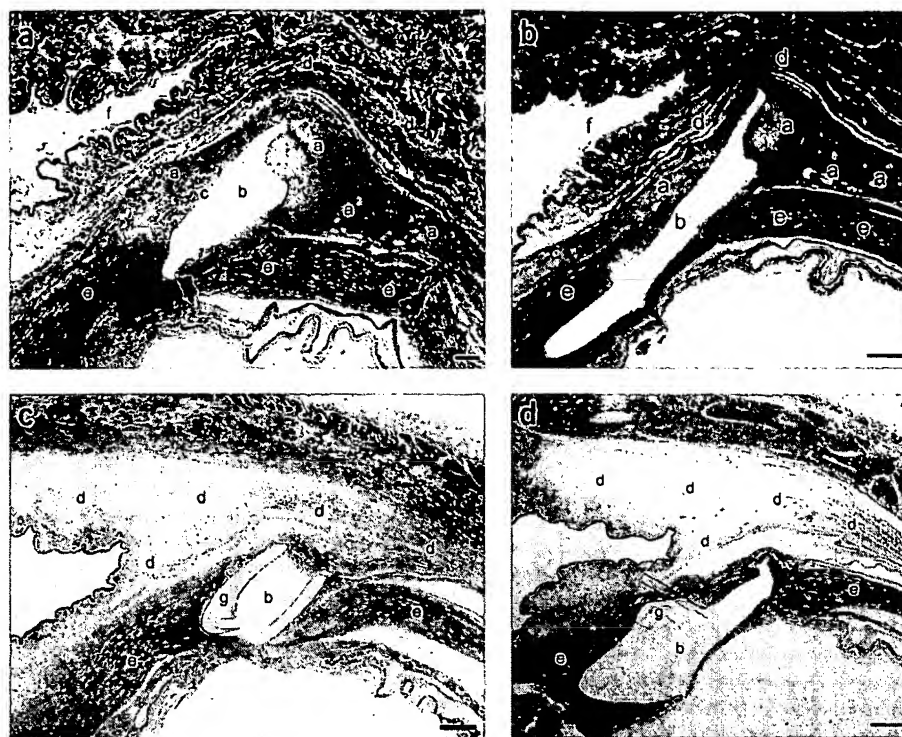
30 d. Histopathological examination of the eyes from rabbits that were successfully treated with dendrimer glucosamine and dendrimer glucosamine 6-sulfate showed minimal scar tissue formation compared to placebo-treated animals (Fig. 6). In addition, there was no evidence of a persistent inflammatory or neoangiogenic response in successfully treated rabbits at day 30.

## DISCUSSION

We have made and optimized two aminosaccharide dendrimer glycoconjugates and used them in combination to safely and synergistically prevent scar tissue formation after eye surgery. The dendrimer glucosamine conjugate acts by inhibiting LPS-TLR4 mediated pro-inflammatory mediator synthesis from immature human DCs and macrophages while allowing activation and maturation of DCs to occur. This is an important new property for a synthetic macromolecule because DC chemokine and cytokine secretion is central to the ability of these cells to orient and amplify specific components of the immune response. Other naturally occurring as well as synthetically derived macromolecules typically stimulate the release of pro-inflammatory cytokines through the TLR4 pathway and also induce

DC/macrophage maturation<sup>24–27</sup>. The dendrimer glucosamine 6-sulfate conjugate inhibited FGF-2-mediated HUVEC proliferation and this translated into a significant antiangiogenic effect in two human model systems of new blood vessel formation. The combined use of dendrimer glucosamine and dendrimer glucosamine 6-sulfate increased the long-term success of glaucoma filtration surgery from 30% to 80% ( $P = 0.029$ ). This study is the first demonstration that simultaneous targeting of pro-inflammatory mediators and neoangiogenesis with synthetic, water-soluble macromolecules with a narrow polydispersity that are neither toxic nor immunogenic, can safely and synergistically prevent scar tissue formation after surgery.

We used carboxylic acid-terminated PAMAM dendrimers because they are predominantly anionic at physiological pH. They are not toxic *in vitro* or *in vivo* compared to amine-terminated cationic PAMAM dendrimers. For example, cationic dendrimers cause substantial changes to red blood cell morphology at  $10 \mu\text{g/ml}$  whereas anionic dendrimers have no such effect at  $2,000 \mu\text{g/ml}$ <sup>12</sup>. Generation 3.5 anionic dendrimers were used because they are not toxic to mice at  $95 \text{ mg/kg}$ , and because they have a therapeutically useful pharmacokinetic profile<sup>12</sup>. Glucosamine and glucosamine 6-sulfate were



**Figure 6** Histological cross-sections of rabbit eyes at day 30. (a,b) Histological cross-sections stained with haematoxylin & eosin and Masson's trichrome, respectively, from a control rabbit eye at day 30 after surgery. Masson's trichrome stains collagen green and muscle red. The sections show that the surgical drainage channel is surrounded by hypercellular scar tissue containing collagen. This extensive scar tissue formation prevents the drainage of aqueous humor across the conjunctiva and leads to the failure of the surgery. (c,d) Histological cross-sections stained with haematoxylin & eosin and Masson's trichrome, respectively, from a dendrimer glucosamine and dendrimer glucosamine 6-sulfate treated rabbit eye at day 30 after surgery. The sections show that the surgical drainage channel and cannula are surrounded by loose connective tissue. There is minimal scar tissue formation. This means that aqueous humor can drain across the conjunctiva and that the surgery has been successful. Normal collagen fibrils are seen in the cornea in all sections. a, scar tissue; b, channel; c, stitch material; d, conjunctiva; e, cornea; f, fornix; g, cannula. Scale bars, 1 mm.

used because several studies have suggested that they may improve wound healing<sup>28–30</sup>. Furthermore, glucosamine is not toxic when administered intravenously and diffuses rapidly from the blood stream into tissues<sup>31–33</sup>. It is also, by far, the most important precursor for all of the modified sugars found in the glycosaminoglycans that make up the backbone of connective tissue proteoglycans. This is also the first demonstration that anionic dendrimer conjugates of aminosaccharides can be made using water-based reactions rather than organic solvents. As such, it has important implications for their potential manufacture as pharmaceutical products.

The dendrimer glucosamine conjugate inhibited the synthesis of pro-inflammatory chemokines and cytokines from LPS treated DCs and MDMs (Fig. 3c,d and Table 1). These dose-dependent effects are consistent with dendrimer glucosamine having an inhibitory effect on the TLR4 that mediates LPS-induced stimulation of DCs and macrophages. This leads to an alteration of the immunological microenvironment that is sufficient to prevent leucocyte proliferation in a MLR (Fig. 4b) while allowing upregulation of costimulatory molecules (Fig. 4a). Our results suggest that costimulatory molecule upregulation can still occur because  $\beta$ -interferon production in response to LPS is not impaired by dendrimer glucosamine. This may reflect the distinct signal transduction pathways that regulate

chemokine/cytokine production (that is, TIRAP and MyD88) and  $\beta$ -interferon production (that is, TRAM and TRIF). Taken together, our results are consistent with a cell surface-mediated mechanism of action of dendrimer glucosamine that does not disrupt the cytoplasmic processing or trafficking of proteins to the cell surface<sup>34,35</sup>.

Although pattern recognition receptors on DCs and macrophages have been primarily studied because of their role in the identification of exogenous molecules (e.g., LPS), it has recently become clear that the same innate immune mechanisms can also be triggered by surgically mediated tissue injury<sup>36,37</sup>. In this context, our results suggest that inhibition of pro-inflammatory chemokine and cytokine release from DCs and macrophages by dendrimer glucosamine can be sufficient to interrupt the inflammatory, proliferative, maturation and remodeling phases of wound healing<sup>38</sup> that lead to excessive scar tissue formation (Fig. 5c). They also suggest that inhibiting pro-inflammatory chemokine release can abort the sequential release of pro-inflammatory cytokines (Fig. 2). The relevance of this observation is that MIP-1 $\alpha$  and MIP-1 $\beta$  (i) are the first chemokines to be released by LPS<sup>15,16</sup> (ii) their release is triggered via TLR4 following the deposition of fibrinogen in the earliest stages of an inflammatory response<sup>39</sup> and (iii) they are the most important mediators of the corneal inflammation and scarring that leads to blindness after herpes simplex type-1 infection<sup>40</sup>.

The dendrimer glucosamine 6-sulfate conjugate has an inhibitory effect on the three most important hallmarks of angiogenesis in man; that is (i) FGF-2 receptor-mediated endothelial cell proliferation and migration (Fig. 5a) (ii) capillary sprouting by normal endothelial cells (Supplementary Table 1 online) and (iii) new blood vessel formation in a model of cell migration and microvessel formation<sup>6,41–43</sup> (Fig. 5b). Evaluation of antiangiogenic activity in adult animals is difficult because endothelial cells proliferate very slowly, if at all, under normal conditions. However, once a pathological stimulus intervenes, the pro-inflammatory and pro-angiogenic chemokine IL-8 activates endothelial cells, increases adhesion molecule expression, and promotes infiltration by inflammatory cells. These include antigen-activated CD4<sup>+</sup> T lymphocytes which stimulate macrophages to produce TNF- $\alpha$ , IL-1 and IL-6 (ref. 44). This completes a positive feedback loop that leads to endothelial cell migration, division and reorganization<sup>45,46</sup>. Our results show that the combination of dendrimer glucosamine and dendrimer glucosamine 6-sulfate acts synergistically to inhibit these very early and critical stages of the pro-inflammatory and pro-angiogenic responses (Fig. 5c). The preferential accumulation of dendrimer-based conjugates in tissues containing inflammatory cells has the added advantage of amplifying this pharmacological synergy<sup>3</sup>.

These synthetically engineered, narrow molecular weight aminosaccharide dendrimer conjugates are therefore an important

new class of therapeutically useful macromolecules for preventing scar tissue formation. They inhibit the polyvalent and cooperative cell surface receptor-ligand interactions that lead to TLR4 and fibroblast growth factor receptor-mediated signalling. As these two receptors play an important role in the pathogenesis of inflammation and angiogenesis respectively in a variety of diseases, dendrimer glucosamine and dendrimer glucosamine 6-sulfate could have clinically useful applications in other areas of medicine and surgery. For example, it is becoming clear that the molecular mechanism responsible for the LPS-TLR4 mediated syndrome of bacterial sepsis is also responsible for mediating the systemic inflammatory response syndrome (SIRS) in other shock-related diseases<sup>47</sup>. Therefore, aminosaccharide dendrimer conjugates could be used to reduce the mortality associated with SIRS in patients after major surgical procedures, burns, acute pancreatitis and in bacterial sepsis. In the case of the last example given, we have unpublished data to show that the synthesis of pro-inflammatory chemokines and cytokines is significantly reduced by dendrimer glucosamine in human blood exposed to *Escherichia coli*. It will now require further detailed studies in appropriate and clinically validated models for each disease to determine the broader applicability of this therapeutic approach.

## METHODS

Use of human blood cells was approved by the ethics committee of Hammersmith Hospitals Trust. Procedures involving animals were carried out in accordance with the UK Animals (Scientific Procedures) Act 1976.

**Synthesis of glycoconjugate.** In brief, Starburst PAMAM dendrimer generation 3.5 (304 mg, -ONa form, MW = 12,931, Dendritech) was allowed to react with D(+)-glucosamine hydrochloride (134 mg, Sigma-Aldrich), or D(+)-glucosamine 6-sulfate (161 mg, Dextra Laboratories) in the presence of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDCI; 557 mg, Lancaster Synthesis) in water at pH 5. Dendrimer glucosamine and dendrimer glucosamine 6-sulfate were purified over Sephadex G-25 (Amersham Biosciences). Endotoxin was removed using Detoxi-Gel columns (AffinityPak Detoxi-Gel, Pierce) so that dendrimer glucosamine and dendrimer glucosamine 6-sulfate contained <0.06 endotoxin unit/ml (that is, European Community standard for water for injection) as determined using the *Limulus polyphemus* amoebocyte lysate assay (Pyroclon). Full details are given in the Supplementary Notes.

**Cellular toxicity.** Dendrimer glucosamine and dendrimer glucosamine 6-sulfate were cultured with the human T-cell line, Sup-T1 and the human monocytic cell line, U937, for 67 h. PBMCs, MDMs, DCs and HUVECs were also cultured with dendrimer glucosamine and dendrimer glucosamine 6-sulfate for up to 5 d and their effect on cell growth and viability determined by cell count (Coulter ZM), trypan blue exclusion and the MTT assay<sup>48</sup>.

**Anticoagulant activity** was determined by measuring the prothrombin time, which uses the extrinsic coagulation system, and the kaolin partial thromboplastin time, which uses the intrinsic coagulation system. Heparin-like activity was determined by measuring Factor Xa activity. Complement-activating activity was determined by measuring C3 and C4.

**Lipopolysaccharide-induced chemokine and cytokine release.** PBMCs, MDMs, immature DCs and CD3+ T lymphocytes were cultured in growth medium containing RPMI 1640, 330 µg/ml L-glutamine, 200 IU/ml penicillin, 200 µg/ml streptomycin and 10% heat-inactivated human serum (Gibco). We cultured  $8 \times 10^5$  cells/ml with dendrimer glucosamine (200 µg/ml) for 1 h at 37 °C and LPS (10 ng/ml; *Salmonella minnesota*, Sigma L9764) added. Supernatants were harvested 21 h later for MIP-1α, MIP-1β, IL-8, TNF-α, IL-1β, IL-6 and IFN-β (enzyme immunoassay (EIA), R & D Systems). mRNA was harvested at 2 h and measured by real time PCR using a Roche Lightcycler. Details of the PCR methods used are given in Supplementary Methods and Figure 2 online.

Dendrimer glucosamine did not bind to LPS or interfere with any of the enzyme immunoassays used. In other experiments, the cells were first cultured

with LPS and dendrimer glucosamine added 2 h or 4 h later. The reversibility of the immuno-modulatory activity of dendrimer glucosamine was determined by culturing 5-d-old MDMs with dendrimer glucosamine for 1 h, washing the cells with PBS, and adding LPS after 1, 2, 4 or 8 h. Culture supernatants were collected 21 h later.

**DC assays.** Immature DCs were prepared from human monocytes by culturing them for 5 d in growth medium containing IL-4 (1,000 U/ml) and granulocyte-macrophage colony stimulating factor (1,000 IU/ml). FACS analysis confirmed they were DCs (that is, CD3-, CD14-, CD16-, CD19-, HLA DR+) with an immature phenotype (that is, CD25-, CD80-, CD83-, CD86-). LPS lead to their maturation as defined by FACS analysis.

The effect of dendrimer glucosamine on LPS-induced maturation of DCs was determined by exposing cells ( $1 \times 10^6$ ) to dendrimer glucosamine (200 µg/ml) for 1 h followed by culture with LPS (10 ng/ml) for 21 h and FACS analysis. The effect of dendrimer glucosamine on the MLR was determined using [<sup>3</sup>H]-thymidine by culturing γ-irradiated stimulator DCs with dendrimer glucosamine (200 µg/ml) for 1 h and then adding allogeneic peripheral blood responder lymphocytes (100,000/well) for 5 d. γ-interferon and β-interferon were measured by EIA and mRNA PCR.

**Neoangiogenesis assays.** HUVECs were plated (passage 3;  $2.5 \times 10^4$  cells/ml) on gelatin-coated plates in M199 medium (Invitrogen) supplemented with 20% human serum and heparin (50 µg/ml). Dendrimer glucosamine 6-sulfate (25–100 µg/ml) was added for 1 h followed by FGF-2 (10 ng/ml; Peprotech) or VEGF (20 ng/ml, R & D Systems) for 3 d. Cell proliferation was determined using [<sup>3</sup>H]-thymidine. A Matrigel assay was used to determine the effect of dendrimer glucosamine 6-sulfate on microtubule formation<sup>6,49</sup>. HUVECs were plated ( $2.5 \times 10^5$  cells/ml) on tissue culture plates coated with Matrigel (Becton Dickinson) and dendrimer glucosamine 6-sulfate was added. The result was read at 18 h. For the placental angiogenesis assay, blood vessels (1–2 mm diameter) were excised from fresh human placentas, cut into fragments and cultured within a fibrin clot for 32 d. New vessel formation was quantified blind twice weekly by three observers<sup>6</sup>. The presence of endothelial cells in new vessels was confirmed using Factor VIII and CD31 antibodies.

**Rabbit glaucoma filtration surgery model of excessive scar tissue formation.** Twenty female New Zealand white rabbits (2–2.4 kg, 12–14 weeks, Charles River) underwent experimental glaucoma filtration surgery<sup>17</sup>. A fornix-based conjunctival flap was raised behind the limbus and a scleral tunnel to the corneal stroma fashioned. A 22-G/25-mm ventron 2 cannula was passed through the tunnel until it was visible in the cornea. Entry into the anterior chamber was achieved by advancing the cannula to the mid-pupillary area where it was trimmed, beveled at its scleral end and fixed to the scleral surface with a nylon suture. The conjunctival incision was closed with two interrupted and one central mattress nylon suture. Dendrimer glucosamine (400 µg/ml) and dendrimer glucosamine 6-sulfate (200 µg/ml) were administered as a sub-conjunctival (100 µl) and intraperitoneal injection (10 ml) in a double-blind, placebo-controlled study on days -2, -1, 0, 1, 2, 3, 4, 7, 9, 11, 14, 17, 21, 24 and 28. The animals were evaluated twice weekly by masked observers. The primary efficacy end point was bleb survival because this is indicative of the long-term patency of the new filtration pathway created (Supplementary Fig. 1). Bleb failure was defined as the appearance of a vascularized, scarred bleb in association with a deep anterior chamber.

All results are shown as the mean ± s.e.m. *P* values were calculated using a Student's *t*-test.

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## COMPETING INTERESTS STATEMENT

The authors declare competing financial interests (see the *Nature Biotechnology* website for details).

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